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Actions of cyclosporin A on cultured rat mesangial cells

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Actions of cyclosporin A on cultured rat mesangial cells. The effects of cyclosporin A (CsA) on planar surface area of cultured rat mesangial cells (PCSA) and cross-sectional area of isolated rat glomeruli (GCSA) were tested. The same experiments were performed after preincubation with platelet activating factor (PAF) antagonists (BN 52021, alprazolam) or calcium channel blockers (verapamil). Areas of cells or glomeruli were analyzed by a computer-assisted method. CsA reduced PCSA in a time-dependent (significant effects began at 15 min, about 12% of reduction with 10^{-6} M CsA) and dose-dependent (no effect at 10^{-9} M CsA, maximal reduction of about 30% at 60 min of incubation with 10^{-7} M CsA) fashion. BN 52021 (5.10^{-5} M) and alprazolam (10^{-5} M) completely inhibited the reduction of mesangial cell area induced by CsA. Verapamil (10^{-5} M) only partially inhibited this action. Glomerular cross-sectional area decreased after 30 minutes of incubation with 10^{-6} M CsA (1.45 ± 0.02 vs. 1.55 ± 0.02 m$^2 \cdot 10^{-8}$, $P < 0.001$), an effect that was inhibited by BN 52021 or verapamil. In addition, 10^{-6} M CsA increased PAF production by isolated rat glomeruli (425 ± 80 pg/mg vs. 198 ± 13 pg/mg in control glomeruli, $P < 0.01$), an effect which was not inhibited by verapamil. These results suggest that CsA could reduce GFR by decreasing the glomerular ultrafiltration coefficient, perhaps as a consequence of the contraction of mesangial cells. PAF seems to play a pivotal role in the genesis of this action.

Cyclosporin A (CsA) probably represents the most important therapeutic advance which has taken place in recent years in the field of kidney transplantation [1]. However, its nephrotoxicity remains a relevant clinical problem [2]. Among the wide spectrum of renal alterations induced by CsA, the production of transient acute renal failure has received special attention [3]. The mechanisms underlying this disorder are not completely known. Acute intravenous injection to rats of doses of CsA similar to those used in human transplantation induces a progressive increase in renal vascular resistances (RVR) accompanied by a parallel reduction in renal blood flow (RBF) and glomerular filtration rate (GFR) [4]. The renin-angiotensin system [5], the autonomous nervous system [6], and some prostanoids [5, 7] have been implied in the genesis of these alterations. Recently, Barros et al [8] have shown that CsA is able to directly modify the glomerular ultrafiltration coefficient (K_f) independently of the changes in RBF, suggesting another physiopathological alternative to the CsA-induced reduction of the GFR.

Several local mediators probably play an important role in the regulation of RVR or K_f [9]. Platelet activating factor (PAF) seems to be one of them, it being able to reduce RBF and GFR [10]. Recent results have shown that isolated glomeruli [11] or cultured rat mesangial cells [12, 13] contract in presence of PAF. In addition, Schlondorff and colleagues have demonstrated that under certain conditions cultured mesangial cells are able to synthesize PAF [14].

In this work, we analyzed the effects of CsA on the contraction of cultured rat mesangial cells and isolated glomeruli. As these effects have been related to changes in the K_f [15, 16], with these experiments we tried to clarify some of the mechanisms responsible for the acute reduction of GFR induced by CsA. Moreover, we attempted to assess a possible role of PAF as a mediator of the observed effects of CsA.

Methods

Materials

Collagenase type IA, from Clostridium histolyticum and L-glutamine were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Penicillin was obtained from Laboratories Level SA (Barcelona, Spain). Streptomycin sulfate was obtained from Antibioticos SA (Madrid, Spain). Hank's balanced salt solution and total calf serum were obtained from Flow Laboratories (Woodcock Hill, UK). Cyclosporin A was by Sandoz (Basel, Switzerland). BN 52021 was from Henri Beaufour Institute (Paris, France). Verapamil was from Knoll (Madrid, Spain) and alprazolam was purchased from Upjohn (Kalamazoo, Michigan, USA). PAF was purchased from Bachem (Bubendorf, Switzerland) and [^{3}H]-Serotonin (5-Hydroxy[G-^{3}H]-tryptamine creatinine sulphate) and [^{3}H]-thromboxane B_2 from Amersham (Buckinghamshire, UK). [^{125}I]—cyclosporine was obtained from Incstar (Stillwater, Minnesota, USA).

Glomerular isolation and mesangial cell culture

Renal glomeruli were isolated from Wistar rats weighing 150 to 200 g and maintained on standard rat chow with free access to tap water. Kidneys were removed under ether anesthesia and glomeruli isolated by successive mechanical sieving (105 and 75 μm) as previously described [17]. The final preparation consisted of glomeruli without Bowman's capsule and afferent or efferent arterioles, with a tubular contamination of less than 5%. Buffer A (Tris 20 mM, NaCl 130 mM, KCl 10 mM, sodium acetate 10 mM, glucose 5 mM, pH 7.45) was used in all the steps of the isolation procedures.

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Isolated glomeruli obtained by a similar mechanical sieving procedure (150 and 50 μm) from rats weighing 100 to 150 g were treated with collagenase, plated in plastic culture flasks and incubated as previously described [18]. The culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (1 mM), penicillin (60 μ/ml), streptomycin sulfate (60 μg/ml), and buffered with Hepes, pH 7.2. Culture media were changed every two days. Epithelial cells grew rapidly whereas mesangial cells grew slowly, with a peak of mesangial cell density between 18 and 22 days. Studies were performed on this interval when epithelial cells were no longer detected in the culture flasks. Identification of the cells was performed by morphological, immunological and functional criteria [19]. Under phase contrast microscopy, all the cells appeared large and stellate. By transmission electron microscopy, the examined cells contained numerous bundles of microfilaments, high density patches and elongated nuclei. Mesangial cells showed evidences of containing myosin fibers and they did not stain for von Willebrand factor. In addition, the cultures did not show activity of angiotensin converting enzyme. No cells with epithelial or endothelial morphological characteristics were detected in the cultures.

Incubation procedure

In every experiment, cells or glomeruli were washed twice, discarding the culture or isolation media and placed in fresh buffer A containing 2.5 mM Ca^{++}. After 15 minutes at room temperature, experiments were started.

In a first set of experiments, cultured mesangial cells were incubated at several times (8, 15, 30, 60 min) with a fixed concentration of CsA (10^{-6} M final concentration). The mesangial cells were incubated for 30 minutes with variable doses of CsA (10^{-6} to 10^{-9} M final concentrations). In a second set of experiments, cells were incubated with BN 52021 (BN) (5.10^{-5} M final concentration) or alprazolam (ALP; 10^{-5} M final concentration). After 10 minutes, CsA (10^{-6} M) was added to the incubation media (time 0), performing incubations at variable times (8, 15, 30, 60 min). In a similar fashion, preincubation with verapamil (VP) (10^{-5} M final concentration) was made. In order to discard a non-specific effect of BN, angiotensin II (10^{-9} M) was added to mesangial cells preincubated for 10 minutes with BN (5.10^{-5} M final concentration), performing incubations for 45 minutes. Controls were introduced in each group with the same final concentration of the vehicles used to solve the reactives (ALP and VP were solved in buffer A. CsA and BN were solved in ethanol, with a final concentration of 0.1%). Moreover, the intrinsic effects of BN, ALP and VP at the same concentrations cited above were tested. The ability of mesangial cells incubated for 60 minutes with CsA 10^{-6} M to exclude the trypan blue dye was also tested.

Isolated glomeruli were incubated for 30 minutes at room temperature with CsA (10^{-6} M final concentration), and compared with glomeruli treated in the same way but preincubated for 10 minutes with BN (5.10^{-5} M final concentration) or VP (10^{-5} M final concentration). As in the case of the cells, controls were performed with the solvent and with BN or VP alone.

Determination of planar cell-surface area (PCSA) and glomerular cross-sectional area (GCSA)

While incubations were performed, mesangial cells grown in conventional plastic culture flasks or samples of 100 μl of glomerular suspensions on excavated glass slides, maintained at room temperature (22 ± 2°C), were observed under phase contrast with inverted Olympus photomicroscope (Olympus IMT2, Shibuya-Hu, Tokyo, Japan) with a 150 magnification [11, 20]. Serial photographs were taken under the experimental conditions cited above. Fifteen to 27 cells and 30 to 50 glomeruli were analyzed per photograph. PCSA and GCSA were determined by computer-aided planimetric techniques (Cardio-80, Kontron Medical, FRG). Actual areas were calculated after correction for microscope and photographic magnifications. Measurements were performed by two investigators in a blind fashion.

Production of PAF by isolated rat glomeruli

Freshly isolated glomeruli were incubated at room temperature for 30 minutes in tubes containing 1.5 ml of buffer A with phenylmethylsulfonyl fluoride (PMSF, 10^{-4} M final concentration), to prevent rapid degradation of PAF. CsA at 10^{-6} M or ethanol (0.2% final concentration), the solvent of CsA, were added to the incubation media. Afterwards, similar incubations were performed but in the presence of 10^{-5} M VP. A blank containing 10^{-6} M CsA but no glomeruli was also introduced. Protein quantitation in aliquots from the glomerular suspension was performed according to Lowry et al [21]. Glomeruli were separated for centrifugation at 3000 rpm for three minutes at 4°C. Supernatants were mixed with 5 ml of cold methanol acidified with acetic acid (49:1, vol:vol), and PAF was extracted as described below. Glomeruli were mixed with 5 ml of cold acidified methanol, sonicated for 30 seconds, and slowly stirred for 30 minutes at 4°C. The tubes were centrifuged and the pellet was again mixed with 5 ml of cold ethanol, stirred for 30 minutes and centrifugated. Both methanolic phases were pooled. PAF was extracted from the methanolic phase as previously described [22]. In brief, chloroform and water were added to the methanol to a proportion of 1:0.9:1 vol:vol (chloroform:water:methanol) and gently stirred. After phase formation by centrifugation, chloroform phase was removed and new chloroform added to the methanol:water in the same proportion as reported above. The process was repeated and both chloroform extracts were pooled and dried under N_2 atmosphere. PAF extraction was performed with silica cartridges (Sep-Pak, Waters, Milford, Massachusetts, USA). Cartridges were rinsed with 5 ml of chloroform, and then the sample resuspended in 5 ml of chloroform acidified with HCl to pH 3 to 4 was applied to the cartridge. The column was eluted sequentially with 5 ml of chloroform-methanol (3:2), 5 ml of chloroform-methanol (1:3) and 5 ml of methanol-water (3:1). The last fraction containing PAF was collected and dried under nitrogen atmosphere. The recovery of PAF in the whole extraction procedure varied between 70 and 80%. When ^{125}I-cyclosporine or ^{3}H-thromboxane B_2 was added to the incubation media, no radioactivity was recovered in this fraction. Bioassayable PAF activity was determined by the release of ^{3}H-serotonin from preloaded rabbit platelets as previously described [22]. A standard curve of response to PAF was made

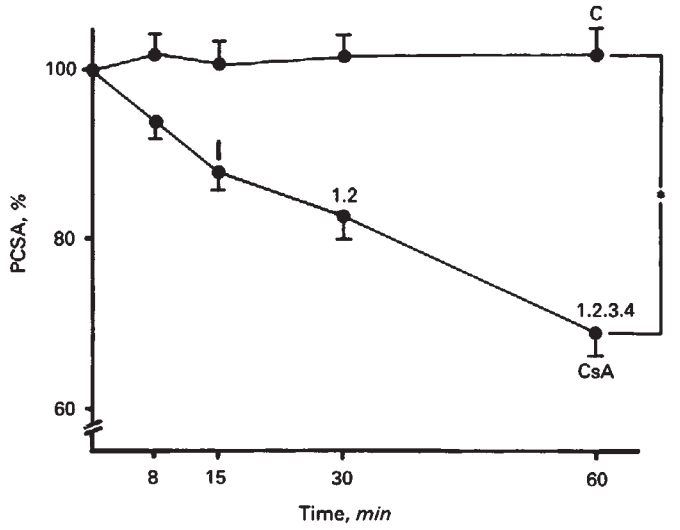

Fig. 1. *Effect of cyclosporin A on the planar cell surface area (PCSA) of cultured rat mesangial cells.* Results are expressed as percent (%) of the initial PCSA (100%) and each point represents the mean of 5 experiments (25–50 cells measured in each experiment). C, control cells; CsA, cells incubated with 10^{-6} M CsA. *$P < 0.05$ vs. C. 1:$P < 0.05$ vs. time 0. 2:$P < 0.05$ vs. time 8. 3: $P < 0.05$ vs. time 15. 4:$P < 0.05$ vs. time 30.

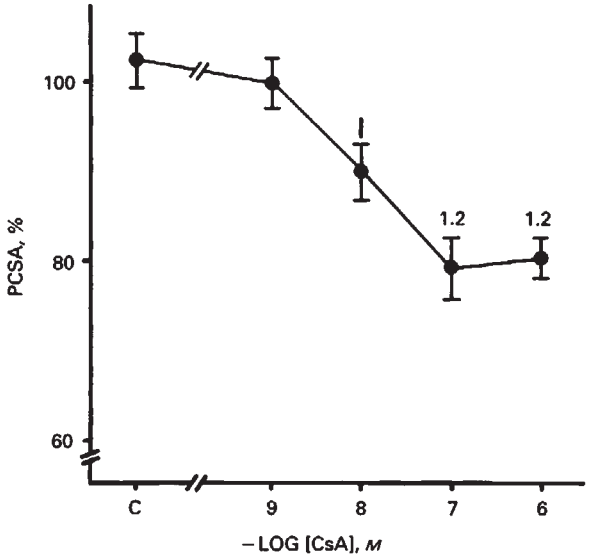

Fig. 2. *Effect of variable doses of cyclosporin A on the planar cell surface area (PCSA) of cultured rat mesangial cells at 30 min of incubation.* Results are expressed as percent (%) of the initial PCSA (100%) and each point represents the mean of 5 experiments (25–50 cells measured in each experiment). C: Control cells. 1:$P < 0.05$ vs. C and 9. 2:$P < 0.05$ vs. 8.

with concentrations between 0.01 ng/ml and 1 ng/ml. The amount of ^{3}H-serotonin released for the lower standard concentration was significantly higher than the nonspecific release. Only the values corresponding to the linear portion of the curve (between 0.01 ng/ml and 0.2 ng/ml) were used for quantitation. Intra-assay variation was about 6.4% and PAF interassay variation was about 17%. PAF production was considered the result of PAF in supernatants plus PAF in glomeruli.

Statistical methods

Results are expressed as $\bar{X} \pm$ SEM. In the studies with cells, comparison were performed by the paired Student's *t*-test, two-way analysis of variance and Scheffe's multiple comparison test. For the glomeruli, the unpaired Student's *t*-test, one-way analysis of variance and Scheffe's multiple comparison test were used. Differences in the glomerular PAF production were analyzed by the Kruskal-Wallis and Mann-Whitney tests.

Results

PCSA decreased when mesangial cells were incubated in the presence of 10^{-6} M CsA (Fig. 1). This effect appeared after 15 minutes of incubation, increasing progressively as a function of the time (Fig. 1). Moreover, it seems that the CsA-induced reduction of PCSA was a dose-dependent phenomenon, being detectable over a concentration of CsA of 10^{-9} M and reaching a maximum at 10^{-7} M (Fig. 2). No changes could be observed in PCSA of control mesangial cells incubated with ethanol through the whole experimental period. Considering a 10% of reduction in PCSA as a significant contraction, 10^{-6} M CsA contracted an average of 76% of cells after 60 minutes of incubation, whereas only 11% of control cells contracted under the same experimental conditions. More than 95% of the cells incubated with 10^{-6} M CsA excluded the trypan blue dye after 60 minutes.

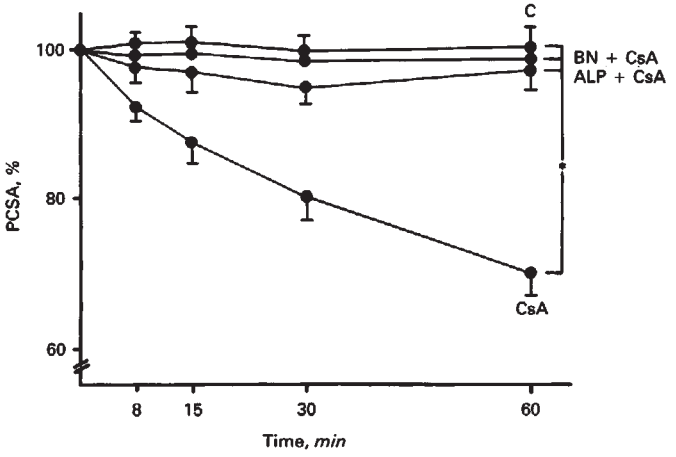

Fig. 3. *Effects of the pre-incubation with BN 52021 or alprazolam on the action of cyclosporin A on planar cell surface area (PCSA) of cultured rat mesangial cells.* Results are expressed as percent (%) of the initial PCSA (100%) and each point represents the mean of 5 experiments (25–50 cells measured in each experiment). C: Control cells. CsA: 10^{-6} M cyclosporin A. BN + CsA: 5.10^{-5} M BN 52021 plus 10^{-6} M cyclosporin A. ALP + CsA: 10^{-5} M alprazolam plus 10^{-6} M cyclosporin A. *$P < 0.05$ versus C, BN + CsA and ALP + CsA.

Preincubation of mesangial cells with BN or ALP completely blunted the contractile response to CsA. In both cases, PCSA did not show consistent variations for 60 minutes with respect to the initial surface, behaving as control cells (Fig. 3). In contrast, when 10^{-9} M angiotensin II was added to mesangial cells preincubated for 10 minutes with BN, a significant reduction of PCSA was observed after 45 minutes of incubation (68 ± 4% of the initial PCSA; $N = 3$). VP was also able to partially inhibit the CsA-induced reduction of PCSA, but a significant

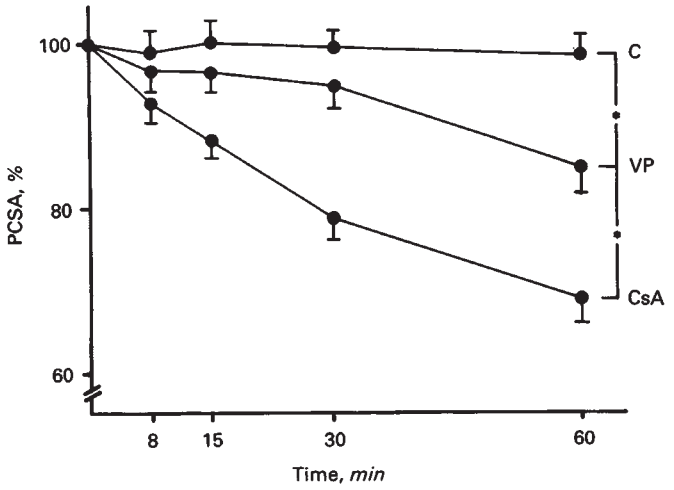

Fig. 4. *Effects of the pre-incubation with verapamil on the action of cyclosporin A on planar cell surface area (PCSA) of cultured rat mesangial cells.* Results are expressed as percent (%) of the initial PCSA (100%) and each point represents the mean of 5 experiments (25–50 cells measured in each experiment). C: control cells. CsA: 10^{-6} M cyclosporin A. VP: 10^{-5} M verapamil plus 10^{-6} M CsA. *$P < 0.05$ vs. C.

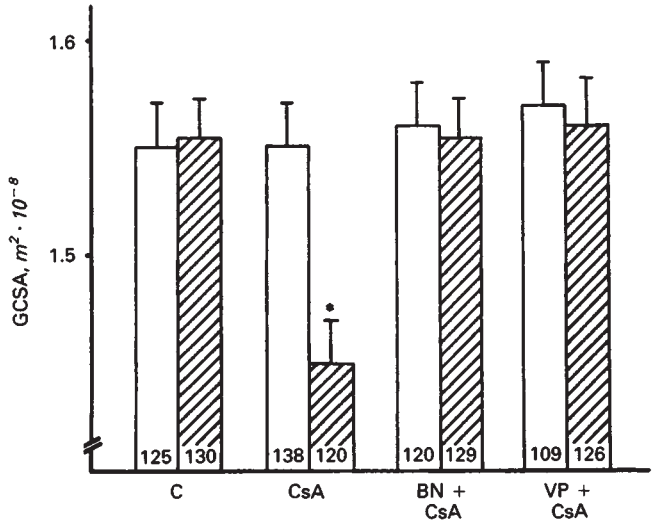

Fig. 5. *Effect of 10^{-6} M cyclosporin A on isolated rat glomeruli pre-incubated with 5.10^{-5} M BN 52021 (BN + CsA) or 10^{-5} M (VP + CsA) verapamil.* Each bar represents the mean of 3 experiments and numbers within the bars are the average number of glomeruli analyzed in each experiment). *$P < 0.05$ vs. time 0 and vs. C, BN + CsA and VP + CsA at time 30.

Table 1. Time course studies of PCSA from cells incubated with BN 52021 (BN: 5.10^{-5} M) alprazolam (ALP:10^{-5} M) and verapamil (VP:10^{-5} M) alone, in comparison with control (C) cells

		Time *min*			
		8	15	30	60
PCSA[a]	C	102 ± 3	101 ± 2	102 ± 3	103 ± 3
	BN	104 ± 5	102 ± 4	104 ± 2	101 ± 4
	ALP	99 ± 2	98 ± 4	101 ± 2	98 ± 5
	VP	98 ± 4	101 ± 3	101 ± 5	97 ± 3

[a] PCSA is expressed as percent of initial values. Data are $\bar{X}$ ± SEM.

Table 2. Production of PAF (pg/mg of glomerular protein) by isolated rat glomeruli

	Control	CsA
Without VP $N = 12$	198 ± 13	425 ± 80[a]
With VP $N = 6$	224 ± 56	401 ± 61[a]

Abbreviations are: CsA, 10^{-6} M cyclosporin A; VP, 10^{-5} M verapamil. Values are $\bar{X}$ ± SEM.
[a] $P < 0.05$ vs. control.

reduction of PCSA was still observed after 60 minutes of incubation (Fig. 4). Neither BN or ALP nor VP modified PCSA when used without CsA (Table 1).

Figure 5 shows the effects of CsA on isolated rat glomeruli. The GCSA at time 0 did not differ among the four experimental groups. However, after 30 minutes of incubation, the mean GCSA of CsA-treated glomeruli decreased significantly with respect to the basal values, whereas no changes could be detected in control glomeruli. Preincubation with BN or VP totally inhibited the reduction of GCSA induced by CsA.

Table 1 shows the glomerular production of PAF with (CsA) or without (Control) cyclosporine in presence or absence of VP. CsA induced a significant increase in the amount of PAF synthesized by isolated glomeruli, an effect which was not inhibited by VP. VP alone did not modify the glomerular production of PAF.

Discussion

The present results demonstrate the ability of CsA to reduce planar surface area of rat mesangial cells in primary culture. This effect is time dependent, appearing after 15 minutes of incubation at room temperature with 10^{-6} M CsA, and increasing progressively. Moreover, a dose-dependent response was observed, with no action of CsA at 10^{-9} M and a maximal reduction of mesangial planar surface at 10^{-7} M. It does not seem probable that the observed effects could be explained by a nonspecific toxic action of CsA on cultured mesangial cells as no detachment of the cells were observed in any case and cells were able to exclude the trypan blue dye after the incubations were performed.

The mechanisms responsible for this reduction of planar surface have been partially studied in the present work. When the effect of CsA was tested in presence of two well-known inhibitors of the interaction of PAF with its receptors, BN 52021 and alprazolam [23], the effect on the planar surface area of mesangial cells was completely abolished. This blockade does not seem to be a nonspecific action of the BN, as this substance was unable to inhibit the cellular contraction induced by angiotensin II. These results suggest that the production of PAF is an intermediate step in the induction of the observed effects of CsA on cultured mesangial cells. In addition, the use of the calcium channel blocker VP [24] partially prevented the reduction of mesangial planar surface area induced by CsA, suggesting that entry of calcium to the cell could play some role in the observed effects of CsA on mesangial cells. These results strongly suggest that the incubation of cultured rat mesangial

cells with CsA stimulates the synthesis of PAF. PAF, acting on the cultured cells, could modify the cytosolic free calcium concentrations, thus determining a cell contraction. Previous experimental results support these hypotheses: 1.) Cultured rat mesangial cells are able to synthesize PAF, at least under adequate stimulus [14]. 2.) Planar surface of cultured mesangial cells is reduced by PAF [12, 13, 25]. 3.) PAF increases the intracytosolic concentration of free calcium [26, 27]. 4.) Calcium channel blockers can inhibit the actions of PAF on planar surface of mesangial cells [25]. 5.) Some studies performed in vivo have described a beneficial role of the PAF antagonists [28] or of calcium channel blockers [29] in the prevention of the CsA-related nephrotoxicity. However, these results do not rule out a possible inhibitory effect of VP on the CsA-induced stimulation of PAF synthesis.

Two aspects of the present work require an additional discussion. It is possible that PAF is not the only mediator involved in the reduction of cell surface induced by CsA. Other mediators could also play a role before or after PAF production. However, the total inhibition of CsA effects on mesangial cells when they were incubated with BN 52021 or alprazolam suggests that the increased synthesis of PAF is a limiting step in the above described actions. The fact that VP only partially reversed the CsA-induced reduction of planar surface of mesangial cells emphasizes the important role of the intracellular calcium pools or other intracellular mediators in the response of mesangial cells to different cellular stimuli [30]. Something similar has been reported for the effect of angiotensin II in mesangial cells [31].

The physiopathological importance of the present results must be carefully analyzed. Reduction of planar surface of mesangial cells has been widely accepted as mesangial contraction [15, 16, 32–34]. However, the present work lacks more specific criteria of cellular contraction [35], and it could be argued that the reduction of planar cell surface does not necessarily mean cellular contraction. In order to analyze the problem with a different approach, the effect of CsA on isolated rat glomeruli was studied. If reduction of planar surface area of cultured mesangial cells really represents cellular contraction, a reduction of GCSA could be expected when glomeruli were incubated under the same conditions as the cells. In fact, this was the result obtained when glomeruli were incubated with CsA 10^{-6} M, an action also inhibited by BN 52021 or VP. Although this was only an indirect approach which did not take into account a possible effect of CsA on epithelial or endothelial glomerular cells, it must be considered a reasonable hypothesis that must be confirmed by additional studies. Some link could be established between these in vitro results and some in vivo evidences. Recent results from Barros et al [8] have shown that CsA could modify the glomerular filtration rate through an effect on the ultrafiltration coefficient (K_f). A relationship between mesangial contraction and reduction of ultrafiltration surface, and consequently K_f, has been proposed as one of the mechanisms responsible for the reduction of the GFR induced by several hormones [15, 16]. Thus, we could suggest that CsA-induced reduction of mesangial planar surface or GCSA could be related to this decrease of K_f.

To confirm the hypothesis that PAF could mediate the observed effects of CsA on cultured mesangial cells and isolated glomeruli of rats, we measured the PAF synthesis by isolated rat glomeruli in presence of CsA. To improve the method of detection, PMSF was added to the incubation media in order to inhibit the cellular acetyl-hydrolase activity [23]. Moreover, we performed a previous extraction of the biological sample, being able to completely separate PAF from thromboxane B_2 or CsA. In these conditions, we could observe a significant increase in the PAF production after incubation with CsA. These results strongly support the role of PAF in CsA-induced glomerular and mesangial contraction. In addition, we tested the effect of VP on the CsA-induced glomerular production of PAF. It did not inhibit the stimulating effect of CsA, suggesting that it partially blocked the contraction of mesangial cells induced by CsA by blocking calcium movements through the cellular membrane, not by preventing the increased synthesis of PAF induced by CsA.

In summary, these results show that CsA reduces planar surface area of cultured mesangial cells and GCSA of isolated glomeruli, suggesting a possible contractile role for CsA at the glomerular level. This fact could be related to the CsA-induced decrease of K_f already reported and could mediate the acute renal insufficiency attributed to CsA [3]. PAF production seems to be a key intermediate step in the genesis of this action of CsA. Although the present results point to calcium channel blockers as an useful tool in preventing this effect of CsA, PAF antagonists seem to be more effective; its pharmacological use deserves further studies.

Acknowledgments

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Note added in proof

In the interim between acceptance and publication of this manuscript, H. Meyer Lehnert and R. W. Schrier have published that cyclosporin A enhances vasopressin-induced contraction of cultured rat mesangial cells (*Kidney Int* 34:89–98, 1988).

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